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Ménage-à-trois: The Amoeba *Nuclearia* sp. from Lake Zurich with its Ecto- and Endosymbiotic Bacteria

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Abstract

We present a fascinating triad relationship between a eukaryotic amoeba and its two bacterial symbionts. The morphological characteristics of the amoeba allowed for a confident assignment to the genus *Nuclearia* (Opisthokonta, Nucleraiidae), but species identification resulted in an ambiguous image. Sequence analysis indicated an affiliation to the species *N. thermophila,* however, several morphological features contradict the original description. Amoebal isolates were cultured for several years with their preferred food source, the microcystin-producing harmful cyanobacterium *Planktothrix rubescens*. Symbioses of the amoeba with ecto- and endosymbiotic bacteria were maintained over this period. Several thousand cells of the ectosymbiont are regularly arranged inside a layer of extracellular polymeric substances produced by the amoeba. It was identified as *Paucibacter toxinivorans* (Betaproteobacteria), which was originally isolated by enrichment with microcystins. We found indications that our isolated ectosymbiont indeed contributed to toxin-degradation. The endosymbiont (Gammaproteobacteria, 15-20 bacteria per amoeba) is enclosed in symbiosomes inside the host cytoplasm and represents probably an obligate symbiont. We propose the name "*Candidatus* Endonucleariobacter rarus" for this bacterium that was neither found free-living nor in a symbiotic association. Nucleariidae are uniquely suited model organisms to study the basic principles of symbioses between opisthokonts and prokaryotes.

Key words: Bacteria-protist symbioses; Ectosymbionts; Endosymbionts; Feeding; Nucleariidae; *Paucibacter toxinivorans*.

Introduction

Two billion years of co-evolution between eukaryotes and prokaryotes has led to the establishment of numerous symbiotic associations (Moya et al. 2008). Essentially, all higher animals and plants live in symbiosis with several, up to thousands of prokaryotic species (Marx 2004; Paracer and Ahmadjian 2000; Ruby et al. 2004). Nevertheless, symbioses with bacteria are not restricted to multicellular organisms. Bacterivorous protists live in intimate contact with bacteria, and this spatial co-occurrence is a potential playground for novel symbioses (Görtz and Brigge 1998; Nowack and Melkonian 2010; Schmitz-Esser et al. 2008). Symbiotic bacteria of protists (Gast et al. 2009) are either attached to extracellular structures (ectosymbionts) or inside the host itself (endosymbionts).

In this article the term symbiosis is used as it was first mentioned in a biological context by Heinrich Anton de Bary in the year 1879 (see Appendix 1 in Paracer and Ahmadjian (2000)). Symbiosis includes any kind of coevolutionary based „living together" of „dissimilar individuals". Thus it comprises the whole spectrum of possible interactions reaching from parasitism to mutualism.

So far, only a few associations of bacteria with protists have been characterized in detail, as symbiotic bacteria are often uncultivable when separated from their hosts. Probably in many cases the appropriate cultivation methods and media for a successful isolation of symbionts have not been found. However, cultivation outside an obligate host has still to be considered challenging or even impossible with the available methods. Nowadays, culture-independent techniques like PCR, cloning and sequencing of marker genes, metagenomics, and fluorescence *in situ* hybridization (FISH) ease at least the identification of symbiotic microorganisms.

Here we present a triad relationship, in which an amoeboid host is associated with endo- as well as ectosymbiotic prokaryotes. The amoeba belongs to the genus *Nuclearia,* the type genus of the family Nucleariidae, which was originally assigned to the class Filosea by

Cann and Page (1979). Based on molecular phylogenetic analyses, nucleariid amoebae are a sister group of Fungi (Brown et al. 2009) belonging to the diverse group of the Opisthokonta (Liu et al. 2009; Steenkamp et al. 2006; Zettler et al. 2001). This phylogenetic position makes them uniquely suited model organisms to study the basic principles of symbiosis between opisthokont eukaryotes and prokaryotes. One typical feature of some *Nuclearia* species is the excretion of extracellular polymeric substances (EPS) that form a mucous sheath around the cell - already observed by Artari (1889). A positive periodic acid-schiff (PAS) staining of the EPS, indicating its polysaccharide character, was shown for *N. radians* (described as *Nucleosphaerium tuckeri* by Cann and Page (1979)), for *N. delicatula* (Cann 1986) and for *N. simplex* (Pernin 1976). The EPS can be colonized by ectosymbiotic bacteria that are arranged regularly a few micrometres from the cell membrane (Cann 1986; Cann and Page 1979; Patterson 1984). So far, endosymbiotic bacteria have been detected in two nucleariid species. *N. radians* harbours endosymbionts that are surrounded by a peribacterial membrane in the cytoplasm (Cann and Page 1979), and the amphizoic *N. pattersoni* has a rickettsial endosymbiont (Dyková et al. 2003).

Besides being already a complex community of three microorganisms, several nucleariid amoebae have a peculiar feeding behaviour with respect to prey ingestion and selection (Artari 1889; Cann 1986; Cann and Page 1979). These *Nuclearia* species can ingest toxic filamentous cyanobacteria as their sole source of food. The herein presented *Nuclearia* species isolated from Lake Zurich (Switzerland) preferentially feeds on the filamentous cyanobacterium *Planktothrix rubescens,* which is the main primary producer in this ecosystem (Posch et al. 2012). *P. rubescens* produces and stores several toxic secondary metabolites intracellular, e.g., microcystins (MCs) that are released into the surrounding when the cell wall is broken. Although the ecological role of MCs is still under debate, they seem to be the primary defence of cyanobacteria against grazers (Blom et al. 2001; Kurmayer and Jüttner 1999). However, some invertebrates (Galanti et al. 2013) and even vertebrates

(Pflugmacher et al. 1998) might detoxify these cyanotoxins by the formation of glutathione-MC conjugates. In contrast to the limited knowledge about eukaryotic detoxification pathways of MCs, efficient biological degradation of toxins was reported for various aquatic bacterial strains - see review by Dziga et al. (2013). A possible biodegradation of MCs was also described for a few xenic (plus bacteria) protistan isolates (Combes et al. 2013; Ou et al. 2005; Zhang et al. 2008). Thus, protists living in close association with bacteria might profit from the presence of prokaryotic symbionts that are able to degrade toxins.

In this study, we give an example of a symbiotic interaction that is formed by three organisms (a microbial „ménage-à-trois"). (i) We present an accurate morphological and phylogenetic analysis of the eukaryotic host. (ii) The phylogenetic affiliation of the two prokaryotic symbionts was determined. (iii) We document the growth of the triple microbial community seen as one single organism as well as the dynamics of each player. (iv) Finally, we discuss the role of the ectosymbiont in the degradation of cyanotoxins, with which amoebae are faced via their toxic food.

Results

Morphological and molecular identification of *Nuclearia* sp. strain N

According to morphological traits (including behaviour), the amoebae were undoubtedly classified to the genus *Nuclearia* (Opisthokonta, Nucleariidae). However, the determination at the species level was ambiguous (Table 1). We documented several mismatches in morphological features of already described species (see the summary in Yoshida et al. (2009)) and our *Nuclearia* sp. strain N (Table 1). Cells of our isolate were often spherical (d = 10 to 37 µm; mean: 16.9 µm; n = 718) while floating freely (Fig. 1A, D, G-I), but adopted a flattened form when attached to a surface (Fig. 1B, C, F). Fine hyaline pseudopodia (filopodia), sometimes branched and knobbed (Fig. 1J), could be elongated and retracted. Filopodia were either evenly distributed around the cell (floating form) or concentrated in the

frontal part during locomotion (attached form). Cells divided by binary fission. Cysts were repeatedly observed when environmental conditions changed rapidly or during starvation (Fig. 1E). Cysts were resistant to desiccation, and excystment was stimulated by adding new medium and prey organisms. The formation of syncytia (i.e., fusion of two or more cells) was rarely observed (Supplementary Fig. S1), but multinucleated cells appeared more frequently at the end of the log-phase (Fig. 1F). The subsequent division of multinucleated syncytia (~2 to 30 nuclei) was also documented. The EPS, only visible after Alcian blue staining (Fig. 1G), was usually present but could be lost during starvation periods. The EPS was colonized by a distinct bacterial morphotype that showed a highly regular arrangement (Fig. 1A-D, G). Amoebae without ectosymbionts or a different colonisation pattern were rarely found (Fig. 1H-I). Two different feeding modes were observed. In one mode, cells used a thick filopodium to engulf the tip of a cyanobacterial filament (consisting of up to 100 single cells). In this way, *P. rubescens* cells were ingested sequentially (Fig. 1D). Alternatively, short fragments of filaments could be ingested by phagocytosis (Fig. 1A). Occasionally, we also observed amoebae that could bend and finally break down a long filament into two parts.

Ultra-structural features investigated by TEM showed that food vacuoles with undigested remains (e.g., cell walls) of *P. rubescens* cells dominated the cytoplasm (Fig. 2A). Outside the eukaryotic cells several ectosymbiotic bacteria colonized the EPS (Fig. 2A). No microtubules supported the filopodia, mitochondria had discoidal cristae, and membrane stacks of the dictyosome showed a regular pattern (Fig. 2B). Endosymbiotic bacteria in the cytoplasm had a typical Gram-negative cell wall and were surrounded by a peribacterial membrane (Fig. 2C, D).

Phylogenetic analysis of 18S rDNA sequences (1804 bp) of our isolate (accession number: HG530253) confirmed a close relationship to *N. thermophila* (99.6 % sequence identity, Fig. 3A). Further, two monophyletic groups (one including the species *N. delicatula*, *N. simplex* and *N. moebiusi,* and the second group including another *N. simplex* and *N.*

pattersoni) were closely related to our isolate (~5 % and ~6% sequence divergence in conserved regions, respectively).

The endosymbiont "*Candidatus* Endonucleariobacter rarus"

Endosymbionts (length: 0.86 µm; n = 125) were initially identified by CARD-FISH as Gammaproteobacteria (probe GAM42a, Supplementary Fig. S2 and S3). We constructed a clone library of 16S rDNA sequences from the *Nuclearia* culture. This library was screened with primers specific for Gammaproteobacteria, resulting in 9 relevant sequences (Fig. 3B): *Pseudomonas fluorescens* (1), *Acinetobacter johnsonii* (1), *Stenotrophomonas acidaminiphila* (1), *S. maltophilia* (3), and a novel deeply branching cluster related to Ectothiorhodospiraceae / *Beggiatoa* (3). CARD-FISH with the oligonucleotide probe SteMal-439 for the detection of *S. maltophilia* gave no positive hybridization signals. Therefore, we focussed on the cluster related to Ectothiorhodospiraceae / *Beggiatoa.* Sequences of this cluster were used as templates to design the specific oligonucleotide probe „CoNuc67" (Fig. 3B, Table 2). This probe was used for CARD-FISH analyses, which resulted in a positive signal for all bacterial cells lying within the *Nuclearia* cytoplasm, even at 70% formamide concentration (Fig. 4A, B and Supplementary Fig. S3).

All recovered sequences of this cluster showed strong similarity (Fig. 3B) to only one public database sequence corresponding to an uncultured bacterium (DQ984555; ~97.5% sequence identity). Otherwise, the closest described relatives to our sequence was in the family Ectothiorhodospiraceae (AY298904 *Ectothiorhodosinus mongolicus* and FR733667 *Ectothiorhodospira shaposhnikovii*), which showed a sequence divergence of about 10 %. Therefore, we propose the establishment of a new genus, i.e., "*Candidatus* Endonucleariobacter rarus" (HG530234) for this endosymbiont.

Identification of ectosymbiotic bacteria

Molecular identification based on 16S rDNA was also done for the dominant rod-shaped bacteria (length: 0.92 µm; n = 125) found in a regular spherical arrangement around *Nuclearia* sp. strain N cells (Fig. 1A-D). Screening of a universal („general") 16S rDNA clone library resulted in 42 full length and 19 partial sequences after chimera check. A cluster of 17 of these sequences was found to be closely related to *Paucibacter toxinivorans* (Betaproteobacteria, Fig. 3B). We first applied CARD-FISH with general oligonucleotide probes (Manz et al. 1992), which resulted in a positive signal for Betaproteobacteria (Supplementary Fig. S4). To confirm that our sequences related to *P. toxinivorans* corresponded to the ectosymbionts, we designed a new oligonucleotide probe „Pauci995" (Table 2, Supplementary Fig. S4) specific for our *P. toxinivorans* sequences and published ones (Fig. 3B). Hybridization with this specific probe resulted in positive signals with the ectosymbionts, even at 70% formamide concentration (Fig. 4C).

Growth of the microbial consortium

Two parallel cultures (N1 and N2) of *Nuclearia* sp. strain N reached the stationary phase after 300 h with ~1.2 x 10^4 cells ml^{-1} (Fig. 5A). Maximal growth rates μ_{max} were 0.34 d^{-1} for N1 and 0.29 d^{-1} for N2, respectively. Regularly arranged ectosymbiotic bacteria (usually several thousand per amoeba) in the EPS were found on ~99 % of amoebae, except during the death phase, when the EPS and ectosymbionts disappeared. We observed a rather conservative median of 11 to 18 endosymbionts per amoeba during the growth phase of both N1 and N2 (Fig. 5B, C).

Isolation of ectosymbionts and bacterial degradation of microcystins (MC)

Our isolated *P. toxinivorans* strain SD41 (HG792253) showed a positive hybridization with the probe Pauci995, and the recovered strain"s 16S rDNA sequence was identical to six

sequences from our clone library (HG530232; HG530236; HG530238; HG530239; HG530243; HG530247). We analysed the growth of the isolate with and without the addition of [Asp^3]MC-LR (Fig. 6). Bacterial cultures with [Asp^3]MC-LR in the medium reached higher ODs after only 40 hours, indicating that bacteria benefited from this cyanotoxin. After 163 hours, we compared the [Asp^3]MC-LR concentrations in bacterial treatments with the ones where we added [Asp^3]MC-LR to the medium but no bacteria. Although the statistical analysis (t-test) showed no significant difference between the MC concentrations in the different treatments, we found a relatively large decrease (1570 µg L^{-1}) of [Asp^3]MC-LR in the treatments with bacteria (Fig. 6), and calculated a degradation rate of 231 µg [Asp^3]MC-LR L^{-1} d^{-1}.

Discussion

Morphological versus molecular identification

Analysing phenotypic traits in combination with genomic information from conserved genes (e.g. 18S rDNA) is needed for a holistic species description of protists (Caron 2013). Morphological features of the isolated filose amoeba revealed striking similarities with described species belonging to the Nucleariidae (Cann and Page 1979). Nevertheless, the comparison of our isolate with all former species descriptions showed an incongruity in at least two morphological features (see summary in Yoshida et al. (2009)). The closest morphological similarity was found between *Nuclearia* sp. strain N and *N. simplex*. However, *N. simplex* differs in two points: it is always uninucleated, and the cell size is usually much larger (~30 µm versus ~17 µm of *Nuclearia* sp. strain N).

To our knowledge, the formation of syncytia in the genus *Nuclearia* has been documented only once, which was for the permanently multinucleated *N. delicatula* (Artari 1889). The fusion of *Nuclearia* sp. strain N cells was rarely observed; however, at the end of the log phase, syncytia were more frequent (Fig. 1F). Thus, multinucleated cells cannot be

considered as an exclusive trait of *N. delicatula* anymore. For *Nuclearia* sp. strain N, the formation of syncytia seemed to be a coordinated process, suggesting that it might be beneficial, e.g. under harsh environmental conditions. During food depletion periods, the larger dimensions of syncytia most probably increased the encounter rate with *P. rubescens* filaments, and syncytia also ingested much longer filaments. A subsequent division of syncytia into uninucleated cells occurred when food was available at saturating levels, again suggesting that syncytia are a response to starvation.

As an exact morphotype-based species determination was not possible, we expected that phylogenetic analyses of the 18S rDNA would result in a stringent determination. Surprisingly, *N. thermophila* was the closest relative of *Nuclearia* sp. strain N (99.6% sequence identity). However, there are several mismatches in the described morphological features of the two „species" (Table 1): *N. thermophila* has no extracellular matrix, does not build cysts nor syncytia, has a different mean cell size, and harbours no bacterial symbionts (Yoshida et al. 2009). One could argue that those characters (Table 1) are very flexible and can easily be lost or gained. However, we kept *Nuclearia* sp. strain N in culture for more than 3 years and did not observe a loss of symbionts, the EPS, or the ability to form cysts and syncytia. There are three explanations for the observed differences between the two isolates. First, they could represent two ecoytpes of the same species that drastically differ in phenotypic features. Second, conditions in which the *N. thermophila* was originally grown differed markedly from our approach. Yoshida et al. (2009) kept the amoebae at 25°C on AF6 medium with traces of sterilized flour, a diet that might affect distinct phenotypic traits. Unfortunately, the original isolate of *N. thermophila* is not in culture and is no longer available (Yoshida, pers. com.). Finally, we are aware that a phylogenetic classification based only on 18S rDNA might not be sufficient to resolve a possible „genetic distance" between *N. thermophila* and *Nuclearia* sp. strain N. However, the fact that sequences of *N. thermophila*,

N. delicatula, *N. simplex*, and our isolate formed a monophyletic cluster in the ML tree is consistent with the morphologically determined closest relatives of *Nuclearia* sp. strain N.

The endosymbiotic bacterium "*Candidatus* Endonucleariobacter rarus"

The endosymbiont was exclusively localized inside peribacterial membranes (symbiosomes; *sensu* Schweikert and Meyer (2001)), whereas food vacuoles merely contained *Planktothrix* remnants and no other bacteria. These observations in combination with the fact that we never observed endosymbiont-free amoebae clearly pointed to an obligate interaction. The 16S rDNA of the endosymbiont affiliated with a so far uncharacterized deep branching cluster of Gammaproteobacteria, which had a sequence divergence of about 10 % to the closest described relative of the genus *Ectothiorhodosinus* (Fig. 3B). The low numbers of endosymbionts inside *Nuclearia* host cells and the possible absence of free-living populations could explain the lack of this organism in public databases. Hence, we propose the establishment of a new genus and a new species for the endosymbiont of *Nuclearia* sp. strain N: "*Candidatus* Endonucleariobacter rarus".

A mutualistic, commensalistic or parasitic endosymbiont?

To further elucidate the symbiotic character, we focused on the growth of both the amoeba and its endosymbiont in parallel (Fig. 5). In the case of a pathogenic interaction, we would have expected an overgrowth of the host by the endosymbiont. However, numbers of endosymbionts per amoeba were stable during the entire growth phase (Fig. 5 B, C). Thus, the two microbial partners seemed to be well synchronized, which points to a mutualistic or commensalistic symbiosis. Nevertheless, the low number of endosymbionts per host cell is rather exceptional for protists with intracellular bacteria, and this fact could have important implications for the stability of this symbiosis. This is particularly true if we consider the tight bottleneck that might occur when bacteria are vertically transmitted and single amoebae get

dispersed (Moya et al. 2008). The resulting genetic drift affecting the genome of the symbiont might be important for the fate of this interaction (Sachs et al. 2011). Hence, it will be interesting to describe this potentially „fast evolving" interaction on a genomic level.

P. toxinivorans strain SD41 and the degradation of cyanotoxins

The regularly arranged bacteria surrounding *Nuclearia* sp. strain N pointed to a coordinated interaction. Since the amoeboid growth followed the typical phases, there was no indication for a negative influence by the closely associated prokaryotes. Phylogenetic analysis showed an affiliation to *P. toxinivoran*s (Fig. 3B), which was originally described to degrade the cyanotoxins MC-LR and -YR (Rapala et al. 2005). These secondary metabolites may act as defence against predators (Blom et al. 2001). Although *Nuclearia* spp. were in direct contact with endotoxins when cyanobacteria were digested, we found no indication that amoebae were harmed by their food. Thus, *Nuclearia* must have developed strategies to cope with MCs. There is evidence that some protists, such as ciliates (e.g. *Paramecium* strains), have protein phosphatases other than PP1 and PP2A, and which are not inhibited by MCs (MacKintosh et al. 1990). However, the protein phosphatases of *Nuclearia* spp. are currently not known. Moreover, amoebae might use multiple drug resistance-like transporters to export toxins into the surrounding medium. Therefore, MCs might be made available for bacteria specialized in their degradation (Dziga et al. 2013). The discovery of ectosymbiotic *P. toxinivorans* supports this theory. To test this, a widely distributed MC variant ([Asp^3]MC-LR) was offered as a substrate to the bacterium, and the presence of this variant was found to increase bacterial growth. Moreover, the observed bacterial induced degradation rate of 231 µg [Asp^3]MC-LR L^{-1} d^{-1} is in accordance to degradation rates of other MCs (96-384 µg L^{-1} d^{-1}) described for *P. toxinivorans* (Rapala et al. 2005). Compared to other strains, *P. toxinivorans* has a moderate capability to degrade MCs. Degradation rates of more than 10"000 µg L^{-1} d^{-1} have been found for *Sphingomonas* species and *Ralstonia solanacearum*,

but also much lower values (1-120 µg L^{-1} d^{-1}) have been described, e.g., for different *Sphingopyxis*, *Sphingomonas*, or *Burkholderia* species (for review see Dziga et al. (2013)).

Conclusions

We present a triad relationship between a eukaryotic amoeba and its two bacterial symbionts. The three microorganisms were successfully identified based on their morphology and a phylogenetic marker, and we give some unique initial insights into this microbial „Ménage-à-trois". Future genomic and proteomic analysis may help to unravel metabolic pathways and interconnections between the three microorganisms.

Description of "*Candidatus* Endonucleariobacter rarus"

Endonucleariobacter rarus. Gr. adj. *endo,* within*;* N. L. fem. n. *nuclearia*, the amoeboid genus *Nuclearia*; N.L. masc. n. *bacter*, a rod; N.L. masc. n. *Endonucleariobacter*, a rod-shaped bacterium living inside the amoeboid genus *Nuclearia*. L. masc. adj. *rarus,* rare, referring to the low number of endosymbionts per amoebal cell.

Rod-shaped bacterium (length: 0.86 µm; n = 125), always surrounded by a vacuolar membrane (symbiosome). Gram negative cell-wall organization. Basis of assignment: 16S rDNA sequence (accession number: HG530234) and positive signal with the general FISH oligonucleotide probes EUBI-III, GAM42a and the specific probe CoNuc67 (5"- ATT GCT ACA CAC TCT GTT ACC G -3", this study). Up to now only identified in the cytoplasm of the *Nuclearia* sp. strain N (accession number: HG530253), isolated from Lake Zurich, Switzerland. Uncultured so far.

Methods

Strains and cultures: *Nuclearia* sp. strain N was isolated from the benthic zone of Lake Zurich, Switzerland (47°19'11.5"N, 8°33'10.1"E) in February, 2011. Monoclonal xenic (i.e. plus bacteria) amoebal cultures were established by washing single cells in sterile water, and kept in Tissue Culture Flasks 25 cm^2 ($TPP_®$) at 18° C on autoclaved mineral water (Cristalp). Cultures were grown under a 12 h light (irradiance of 5-15 µmol m^{-2} s^{-1}) / 12 h dark cycle. Axenic cultures of the cyanobacterium *Planktothrix rubescens* BC 9307 isolated from Lake Zurich (Blom et al. 2001; Walsby et al. 1998) served as sole food source for the amoebae. Batch cultures (10 ml) of amoebae were fortnightly renewed by adding cyanobacterial stock cultures (~1 ml) to new culture medium inoculated with 200 µl of an older culture. *Nuclearia* sp. strain N has been deposited in the Culture Collection of Algae and Protozoa (CCAP; http://www.ccap.ac.uk/) with the strain number CCAP 1552/5.

Sequencing of the 18S rDNA (*Nuclearia* sp. strain N): Aliquots (1.5 ml) of dense *Nuclearia* cultures were centrifuged (15 min; 16000 x g), pellets were subjected to several freeze-thaw cycles, and DNA was isolated with the GenElute^{TM} Bacterial Genomic DNA Kit (Sigma). The 18S rDNA was amplified by PCR with the eukaryote-specific primers Euk328f and Euk329r (Moon-van der Staay et al. 2001), PCR products were purified with the QIAquick PCR Purification Kit (Qiagen), and subsequently sequenced with ABI BigDye chemistry on an ABI 3130x Genetic Analyzer (Applied Biosystems). Additionally, the primers SR4f and SR8r (Nakayama et al. 1998) were used for sequencing, and partial sequences were assembled with the software DNA Baser v3.5.0 (Heracle BioSoft).

Clone library and sequencing of 16S rDNA (symbionts): Four hundred individuals of *Nuclearia* sp. strain N were picked with a micropipette, washed in sterile water, centrifuged

(30 min; 16000 x g), and DNA was extracted either by several freeze-thaw cycles or with the GenElute ^{TM} Bacterial Genomic DNA Kit (Sigma). Bacterial 16S rDNA was amplified with primers GM3f and GM4r (Muyzer and Ramsing 1995), products were purified, pooled, and concentrated with the DNA Concentrator Kit (Epigentek). PCR products were cloned into competent *Escherichia coli* cells using a pGEM®-T Vector (Promega) according to the manufacturer‟s instructions. The obtained clone library was screened with plasmid primers (general screening) and additionally with a two-step nested PCR approach (Boutin et al. 2012) with a second primer pair specific for Gammaproteobacteria (Klein et al. 2007). These primers were slightly modified by the insertion of wobbles (S-Sc-gProt-0382-a-S-18_f_wobble 5‟-AGGCAGCAGTGGGGAATM-3‟ and S-Sc-gProt-0946-a-A-18_r_wobble 5‟-GCCCCCGTCAATTCMTTT-3‟). Positive clones were purified with a GenElute™ Five-Minute Plasmid Miniprep Kit (Sigma) and sequenced as above with primers GM3f, GM4r, and GM1f (Muyzer and Ramsing 1995). Partial sequences were assembled and chimeric sequences were detected with the software Mallard and Pintail (Ashelford et al. 2005). Screening with a second primer pair specific for Gammaproteobacteria showed that only 9 sequences of 28 positive clones (Fig. 3B) indeed belonged to Gammaproteobacteria, i.e., *Stenotrophomonas maltophilia* (3), *Stenotrophomonas acidaminiphila* (1), a novel deeply branching cluster related to Ectothiorhodospiraceae / *Beggiatoa* (3), *Pseudomonas fluorescens* (1), and *Acinetobacter johnsonii* (1).

Phylogenetic analyses: Analyses were performed with the ARB software (Ludwig et al. 2004) using the SILVA database SSU Ref 111 (Pruesse et al. 2007). The 18S rDNA sequence from *Nuclearia* sp. strain N was first automatically aligned (FAST_ALIGNER tool) to the sequence of *N. thermophila* (AB433328), and all available complete 18S rDNA sequences of described *Nuclearia* spp. were subsequently added. This allowed for proper alignment of the conserved regions, but long inserts that are typical for Nucleariidae had to be aligned

manually. The beginning and ends of all alignments were trimmed to the length of the shortest sequence.

Bacterial 16S rDNA sequences were aligned with the SINA web aligner (www.arb-silva.de/aligner/) and manually refined. Only full-length sequences (*E. coli* position 8 – 1507) from the clone library and related sequences from the ARB database (quality scores ≥ 88) were used for calculation of the phylogenetic tree.

Maximum Likelihood (ML) and Bayesian inference (BI) methods were used for the phylogenetic reconstructions. Two bootstrapped ML trees (*Nuclearia* spp. and clone library) were calculated (1000 iterations) using the RAxML algorithm on a dedicated web server (Stamatakis et al. 2008) using the GTR (General Time Reversible) model with a Γ distribution for rate heterogeneity among sites, with 4 discrete rate categories. Additionally BI consensus trees were constructed (4 chains; 100000 generations) using the ExaBayes software package (©The Exelixis Lab). The posterior probabilities from the BI trees were added to the ML trees where identical topography for both methods was found. All complete 16S rDNA sequences from the clone library and the 18S rDNA sequence of *Nuclearia* sp. strain N have been deposited in the EMBL database with accession numbers HG530231-HG530253 and HG973425-HG973449.

CARD-FISH and probe design: Two CARD-FISH probes (Table 2) specific for *Paucibacter toxinivorans* and for a novel cluster of Gammaproteobacteria were designed with the dedicated ARB tools. The resulting probes were tested *in silico* in the Ribosomal Database Project (RDP) (www.rdp.cme.msu.edu), and hybridization efficiencies and mismatch stability were analysed with the web tool Mathfish (Yilmaz et al. 2011). The newly designed oligonucleotide probes were tested on *Nuclearia* sp. cultures at different formamide concentrations until highest stringency was achieved (Table 2). Additionally the already published (Manz et al. 1992) general probes EUB I-III, BET42a, GAM42a (Supplementary

Fig. S3 and S4) and the specific probe SteMal-439 (Piccini et al. 2006) were used. The probe NON338 was applied to check for non-specific staining (Supplementary Fig. S2). The best preservation of amoeboid and bacterial morphologies was determined by testing different fixatives (i.e., formaldehyde, Lugol‟s solution, glutaraldehyde). Finally, cells were fixed on ice with Lugol‟s solution (0.5% final. conc.), formaldehyde (2% final. conc.), and followed by decolourization with a few drops of Na-thiosulfate (3% stock solution). Samples were filtered onto white polycarbonate filters (0.2 µm pore size, Millipore) placed on a support filter (Sartorius). Filters were rinsed with distilled water, air dried, and stored at -22° C until further processing. CARD-FISH with fluorescein- and, in case of double hybridization, with Alexa546-labeled tyramides was done for filter sections as described by Sekar et al. (2003). Hybridization took place at 35° C on a rotation shaker for a minimum of 2 h. After hybridization filter sections were counterstained with DAPI (1 µg mL^{-1}).

Microscopy and photographic documentation: Living specimens were observed with differential interference and phase contrast with an Axio Imager.M1 microscope (Zeiss). The extracellular polymeric substances (EPS) of amoebae were visible in brightfield microscopy after staining with Alcian Blue (Logan et al. 1994). CARD-FISH preparations were analysed with epifluorescence on the same microscope. The following optical filter sets (Zeiss) were used: set 01, set 10, set 14, set 43. All photographs were taken with a Canon EOS1000D controlled by the software AxioVision 4.8.2 (Zeiss).

Transmission electron microscopy (TEM): Cells of *Nuclearia* sp. strain N cultures were fixed on ice for 1 h with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.6) followed by embedding in low melting agar (2 %). After cutting the agar block in small pieces (~2-3

mm^3), cells were washed by exchanging the cacodylate buffer (3X) and postfixed for 1 h with 1 % osmium tetroxide (OsO_4). Subsequently, samples were dehydrated in an acetone series (30 %, 50 %, 75 %, 90 % and 100 %) and embedded in Spurr"s resin. Ultrathin sections were made with an Ultracut UCT (Leica) and stained with 1 % (aq.) uranylacetate for 6 min and lead citrate (Venable and Goggeshall 1965) for 4 min. TEM preparations were analysed with a Zeiss EM 10 at 60 kV (Schweikert and Meyer 2001).

Growth curves: We monitored the growth of *Nuclearia* sp. strain N in a duplicate setup (N1 and N2) simultaneously with their per cell number of endosymbiotic bacteria during a 3-week period. In addition, cell sizes of living amoebae were measured interactively with an image analysis system (AxioVision 4.8.2, Zeiss) and distributions patterns of ectosymbionts in the EPS were documented. Cultures were grown in Tissue Culture Flasks 75 cm^2 (TPP$_®$) in 100 ml of autoclaved mineral water (Cristalp). *P. rubescens* was added as food source at the beginning (10 ml of stock culture), together with an inoculum of *Nuclearia* sp. strain N (~130 cell ml^{-1}). We determined abundances of amoebae from live counts in 6 drops of 15 µl ($n \geq$ 100 individuals counting^{-1}) on a daily basis. Aliquots (1.5 ml) for CARD-FISH preparations were taken daily from day 3 to day 21. The number of endosymbiotic bacteria was counted after hybridization with probe GAM42a (Manz et al. 1992) in 24 to 84 amoebae per preparation (total n of inspected *Nuclearia* sp. = 2952).

Isolation of ectosymbionts and bacterial degradation of microcystins: Single *Nuclearia* sp. strain N cells with a densely colonized EPS were picked with a micropipette under a dissection microscope, transferred onto R2A plates (Reasoner and Geldreich 1985) and incubated at 18° C. After several days bacterial colonies were repeatedly picked and streaked on fresh agar plates to obtain clonal isolates of different bacterial strains. CARD-FISH with

the probe Pauci995 (Table 2) allowed for a screening of isolates. One isolate with positive hybridization signal was subsequently grown in liquid R2 medium. DNA was extracted from the liquid culture with the GenElute ^{TM} Bacterial Genomic DNA Kit (Sigma) and the 16S rDNA gene was amplified, purified, and sequenced as described above (*P. toxinivorans* strain SD41, accession number: HG792253). Pure cultures of the isolate were cryopreserved (30 % glycerol, -80° C).

Cells were re-grown on R2A plates until visible colonies appeared. One colony was picked and transferred to liquid R2 medium and grown to a density of ~4 x 10^8 cells ml^{-1}. For the experiment, cells were grown in R2 medium diluted (1:9) with artificial lake water (ALW) medium (Zotina et al. 2003) that was amended with vitamins (thiamine 0.593 µM, niacin 0.08 µM, cobalamine 0.000074 µM, para-amino benzoic acid 0.005 µM, pyridoxine 0.074 µM, pantothenic acid 0.081 µM, biotin 0.004 µM, folic acid 0.004 µM, myo-inositol 0.555 µM). The microcystin variant [Asp^3]microcystin-LR (abbreviated as [Asp^3]MC-LR) was isolated in high purity (> 99 %, HPLC) from *Microcystis aeruginosa* PCC 7806. HPLC purification was performed on a Shimadzu 10AVP system equipped with a photodiode array detector (PDA) on a C-18 Grom-Sil 120 ODS-4 HE reversed phase column (Stagroma, Germany), using solvent A: UV-treated H_2O containing 0.05% trifluoroacetic acid (TFA, Merck) and solvent B: acetonitrile and 0.05% TFA. A gradient was achieved by applying linear increases in three steps (solvent B from 30 % to 35% in 10 min, from 35 % to 70 % in 30 min, and from 70% to 100% in 2 min). Further details on the purification process are given in Blom and Jüttner (2005).

In four parallel experimental set-ups, ~6 x 10^4 cells of *P. toxinivorans* strain SD41 were inoculated to 200 µl of R2 and ALW medium (1:9) containing pure [Asp^3]MC-LR (20.4 mg l^{-1} final concentration). Eight bacterial control treatments were prepared in the same way but without the addition of [Asp^3]MC-LR. Three additional replicates contained only 200 µl medium and 20.4 mg l^{-1} of [Asp^3]MC-LR but no bacterial inoculum, to check for any

contamination or chemical degradation of the toxin during the incubation period. All treatments were incubated at 26° C for 163 h and optical densities (OD at 600 nm) were recorded every 30 min with a microplate reader (SpectraMax 190, Molecular Devices). At the end of the experiment, 150 µl each of the 4 replicates with bacteria and of the 3 controls (only medium and [Asp^3]MC-LR) were diluted with methanol to achieve a 60 % aqueous MeOH solution. For the quantification of [Asp^3]MC-LR, calibration curves were established using a Hydrosphere C18 column (YMC, 4.6 x 250 mm, Stagroma, Switzerland). The specific molar absorption coefficient of [Asp^3]MC-LR was used to prepare accurate standard solutions between 1 and 10 µg (Blom et al. 2001). Quantification was based on the peak area recorded at 239 nm on a HPLC system (Shimadzu 10AVP) equipped with a photodiode array detector (PDA).

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Figure Legends

Figure 1. *Nuclearia* sp. strain N isolated from Lake Zurich, Switzerland. (**A**) Spherical shape ingesting a short cyanobacterial filament (*Planktothrix rubescens*) surrounded by well-arranged bacteria in the extracellular matrix, (**B**) and (**C**) flattened body form attached on surfaces, (**D**) two amoebae feeding on the same cyanobacterial filament, (**E**) two cysts with still intact extracellular matrix, (**F**) multinucleated (3 nuclei) syncytium, (**G**) Alcian blue staining of the extracellular matrix, (**H-I**) different colonization patterns of ectosymbiotic bacteria, (**J**) fine hyaline pseudopodia (filopodia), sometimes branched and knobbed. (**C, I, J**) Phase contrast pictures, all others: differential interference contrast (DIC) pictures. Scale bar: 20 µm in (**A-J**).

Figure 2. Transmission electron micrographs of the amoeba *Nuclearia* sp. strain N. (**A**) Whole cell overview showing several food vacuoles (Fv) with partly digested cyanobacterial material. Note the two endosymbiotic bacteria (En) in the upper part of the cytoplasm. The extracellular matrix is transparent in TEM preparations (low electron density). A few ectosymbiotic bacteria (Ec) arranged around the amoebae are visible. (**B**) Detailed view of the cytoplasm of the amoeba, showing several mitochondria (Mt) with discoidal cristae and parts of the dictyosome (Di). (**C-D**) Detailed views of endosymbiotic bacteria (En) in symbiosomes, i.e. surrounded by peribacterial membranes.

Figure 3. Phylogenetic analysis of the amoeboid host and its ecto- and endosymbiotic bacteria. Bootstrapped Maximum Likelihood (ML) trees (1000 iterations) with posterior probabilities from the Bayesian inference (BI) consensus trees (4 chains, 100000 generations). Node labels: ML bootstrap in %/BI posterior probabilities. Incongruent topology is indicated with ---: (**A**) 18S rDNA of *Nuclearia* ap. strain N isolated from Lake Zurich (Switzerland) related to all published *Nuclearia* spp. sequences with *Candida* as outgroup. (**B**) 16S rDNA phylogeny of sequences from the clone library together with related sequences from the Silva database and *Verrucomicrobia* as outgroup. Clusters with sequences from the symbionts are

shown in detail. On the left hand higher taxonomic affiliations are indicated. Numbers in the brackets represent the number of sequences from the clone library affiliated with the respective cluster (# general screening / # gamma-screening). Curly brackets show the coverage of the CARD-FISH probes Pauci995 and CoNuc67. Scale bars: number of nucleotide substitutions per site.

Figure 4. CARD-FISH double hybridization of four *Nuclearia* sp. strain N individuals. (**A**) DAPI staining, (**B**) hybridization of endosymbiotic bacteria with the probe CoNuc67 and fluorescein-labelled tyramides, (**C**) hybridization of ectosymbiotic bacteria with the probe Pauci995 with Alexa546-labelled tyramides, (**D**) overlay of (**A-C**). Note the strong auto-fluorescence of ingested cyanobacterial filaments in three amoebae in (**B-D**). Scale bars: 20 µm.

Figure 5. Growth of *Nuclearia* sp. strain N and its endosymbiotic bacteria. (**A**) Growth curves of two replicate amoebal cultures (N1 and N2). Dashed lines indicate the succession of the four growth phases: lag, log, stationary and death phase. Shown are means ± standard errors, $n \geq 100$ cells sample^{-1}. (**B, C**) Numbers of endosymbiotic bacteria determined form CARD-FISH preparations with probe GAM42a in N1 and N2, respectively. n = 24 to 84 inspected amoebae sample^{-1}. Boxes indicate the 25th to 75th percentiles, whiskers the 10th to 90th and crosses the 5th and 95th percentiles.

Figure 6. Growth dynamics (optical density values at 600 nm) of the isolated ectosymbiotic bacterium *Paucibacter toxinivorans* strain SD41 with and without the addition of [Asp^3]microcystin-LR to the culture medium. Additionally, three control runs containing only the medium and [Asp^3]microcystin-LR but no bacteria were measured. Bars show the difference of microcystin concentrations after 163 h in the treatments with and without bacteria. Shown are means ± standard errors.

Table1

Table 1. A comparison of the features of *Nuclearia* sp. strain N versus those described for *Nuclearia thermophila.* Note that the two isolates show 99.6 % sequence identity for 18S rDNA (1804 bp). Morphological features in bold indicate differences between the two isolates.

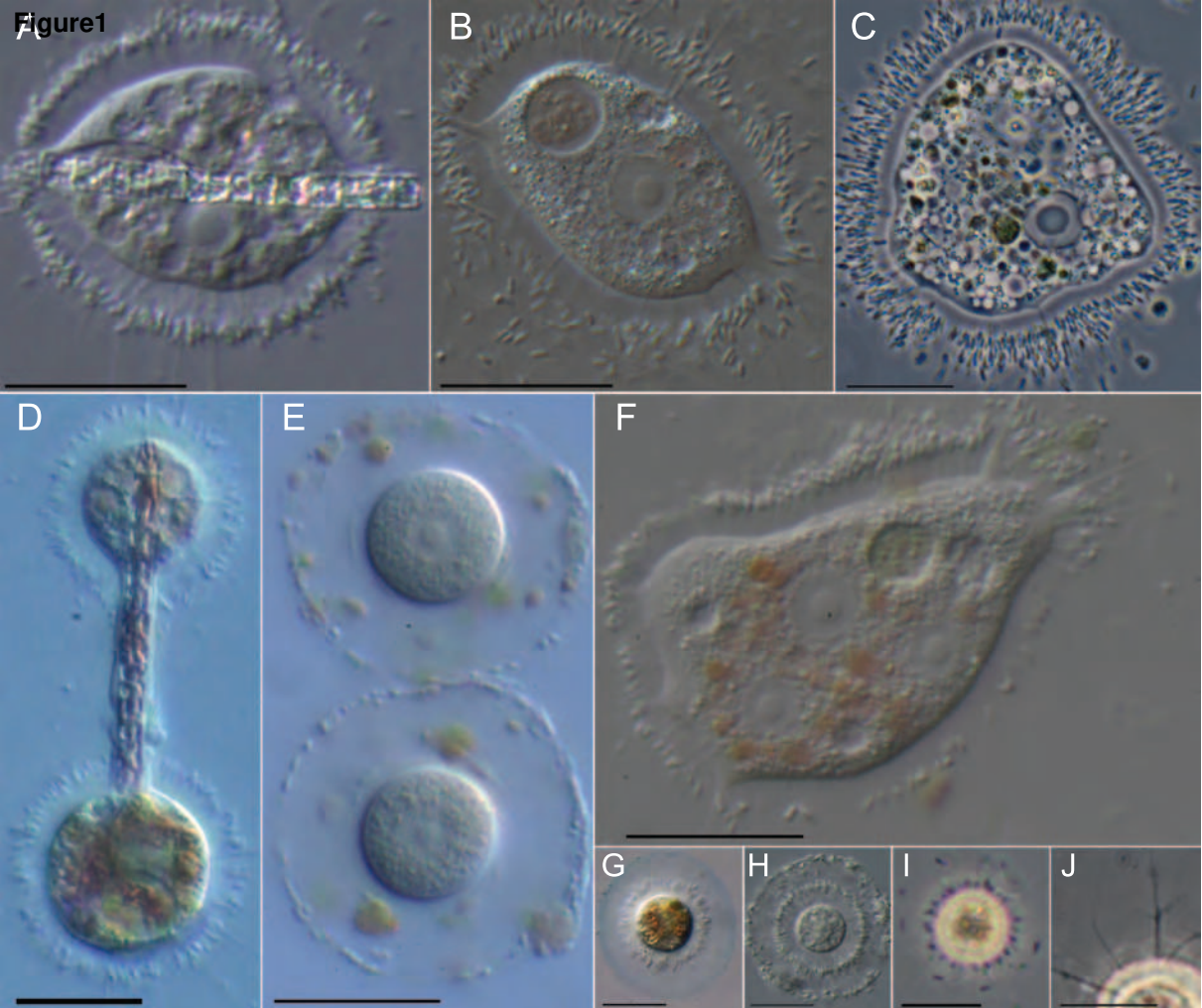
	Nuclearia sp. strain N	*Nuclearia thermophila*
Original description	This study	Yoshida et al. (2009)
Spherical form*	Observed	Described
Flattened form*	Observed	Described
Nucleus*	Uninucleate, **Multinucleate syncytia**	Uninucleate
Nucleolus*	Evident	Evident
Extracellular matrix*	**Observed** (polysaccharide mucous layer)	No matrix
Cyst producing*	**Observed**	Not described
Branching of filopodia*	Observed	Described
Typical body diameter*	16.9 µm (n=718, spherical), 28.6 µm (n=424, spherical with ectosymbionts)	25-40 µm (spherical), 30-65 µm (elongated)
Registered SSU rDNA sequence*	HG530253	AB433328
Ectosymbiotic bacteria	**Observed** (several hundred per amoeba)	Not described
Endosymbiotic bacteria	**Observed** (15-20 bacteria per amoeba)	Not described
Formation of syncytia	**Observed**	Not described
Other features*	Knobbed filopodia	Knobbed filopodia

* These features were listed in Table 1 in Yoshida et al. (2009) for the description of *N. thermophila*.

Table 2. Oligonucleotide probes designed in this study. FA% = Formamide concentration.

Probe	Specificity	Sequence (5' to 3')	FA %
Pauci995	*Paucibacter toxinivorans*	AATCTCTTCGGGATCTCTGACATG	70
CoNuc67	„*Candidatus* Endonucleariobacter rarus“	ATTGCTACACACTCTGTTACCG	70

Figure1



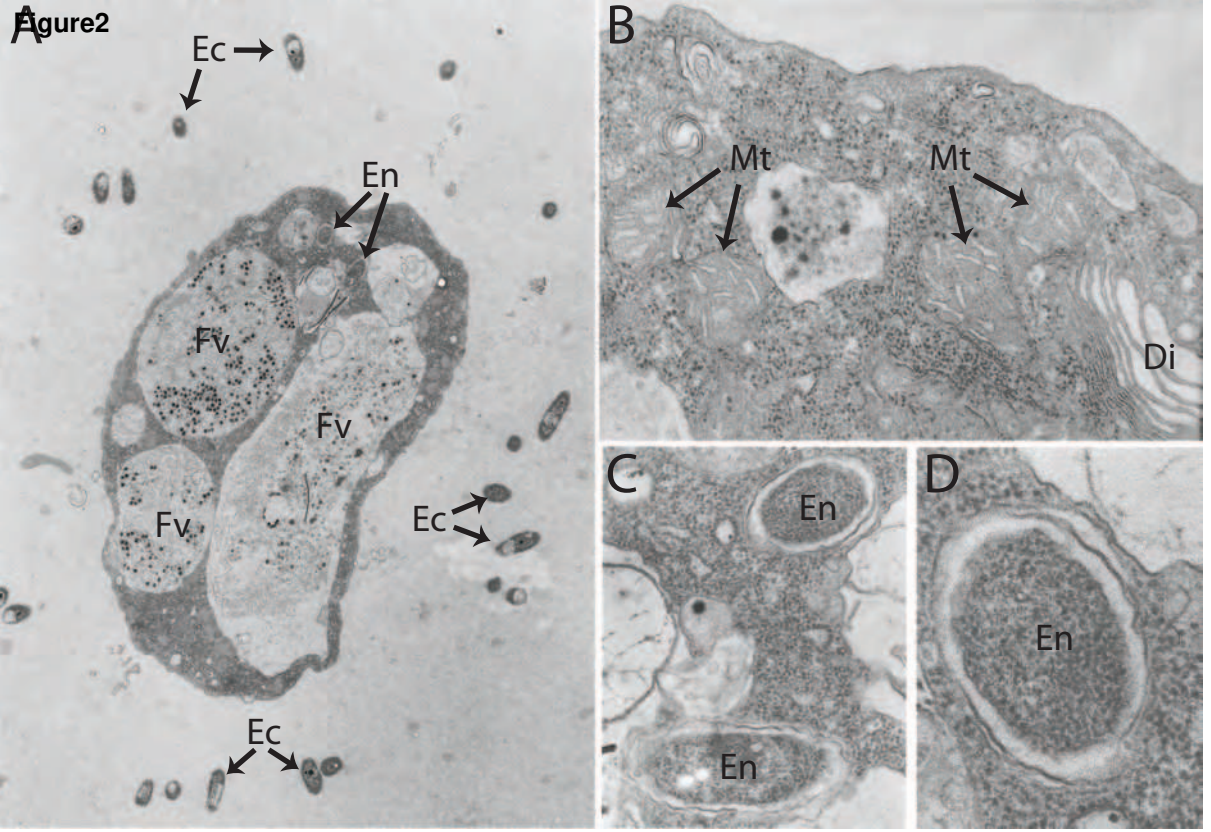


Figure2
A
Ec
En
Fv
Fv
Fv
Ec
Ec
B
Mt
Mt
Di
C
En
En
D
En

Figure3

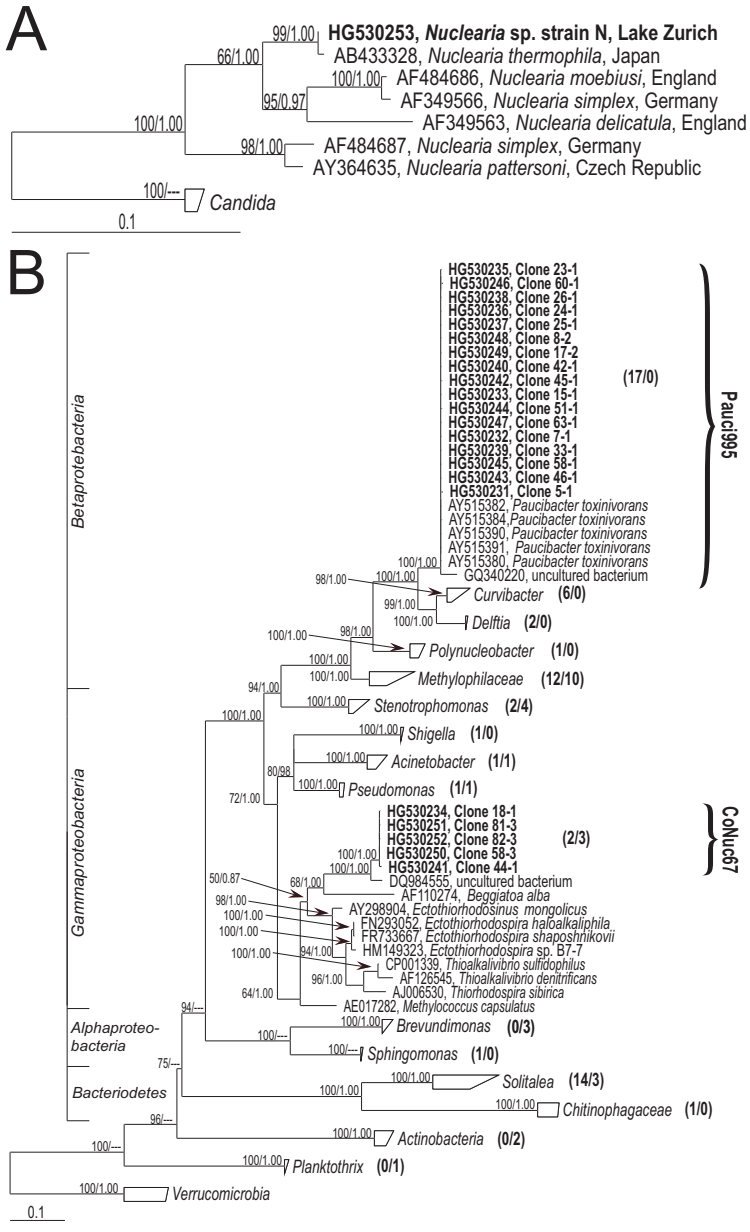

A
99/1.00
HG530253, Nuclearia sp. strain N, Lake Zurich
66/1.00
AB433328, Nuclearia thermophila, Japan
100/1.00
AF484686, Nuclearia moebiusi, England
95/0.97
AF349566, Nuclearia simplex, Germany
AF349563, Nuclearia delicatula, England
100/1.00
98/1.00
AF484687, Nuclearia simplex, Germany
AY364635, Nuclearia pattersoni, Czech Republic
100/---
Candida
0.1
B
Betaproteobacteria
Gammaproteobacteria
Alphaproteo-bacteria
Bacteriodetes
HG530235, Clone 23-1
HG530246, Clone 60-1
HG530238, Clone 26-1
HG530236, Clone 24-1
HG530237, Clone 25-1
HG530248, Clone 8-2
HG530249, Clone 17-2
HG530240, Clone 42-1
HG530242, Clone 45-1
HG530233, Clone 15-1
HG530244, Clone 51-1
HG530247, Clone 63-1
HG530232, Clone 7-1
HG530239, Clone 33-1
HG530245, Clone 58-1
HG530243, Clone 46-1
HG530231, Clone 5-1
(17/0)
Pauci995
AY515382, Paucibacter toxinivorans
AY515384,Paucibacter toxinivorans
AY515390, Paucibacter toxinivorans
AY515391, Paucibacter toxinivorans
AY515380, Paucibacter toxinivorans
GQ340220, uncultured bacterium
Curvibacter (6/0)
Delftia (2/0)
Polynucleobacter (1/0)
Methylophilaceae (12/10)
Stenotrophomonas (2/4)
Shigella (1/0)
Acinetobacter (1/1)
Pseudomonas (1/1)
HG530234, Clone 18-1
HG530251, Clone 81-3
HG530252, Clone 82-3
HG530250, Clone 58-3
HG530241, Clone 44-1
(2/3)
CoNuc67
DQ984555, uncultured bacterium
AF110274, Beggiatoa alba
AY298904, Ectothiorhodosinus mongolicus
FN293052, Ectothiorhodospira haloalkaliphila
FR733667, Ectothiorhodospira shaposhnikovii
HM149323, Ectothiorhodospira sp. B7-7
CP001339, Thioalkalivibrio sulfidophilus
AF126545, Thioalkalivibrio denitrificans
AJ006530, Thiorhodospira sibirica
AE017282, Methylococcus capsulatus
Brevundimonas (0/3)
Sphingomonas (1/0)
Solitalea (14/3)
Chitinophagaceae (1/0)
Actinobacteria (0/2)
Planktothrix (0/1)
Verrucomicrobia
0.1

Figure4

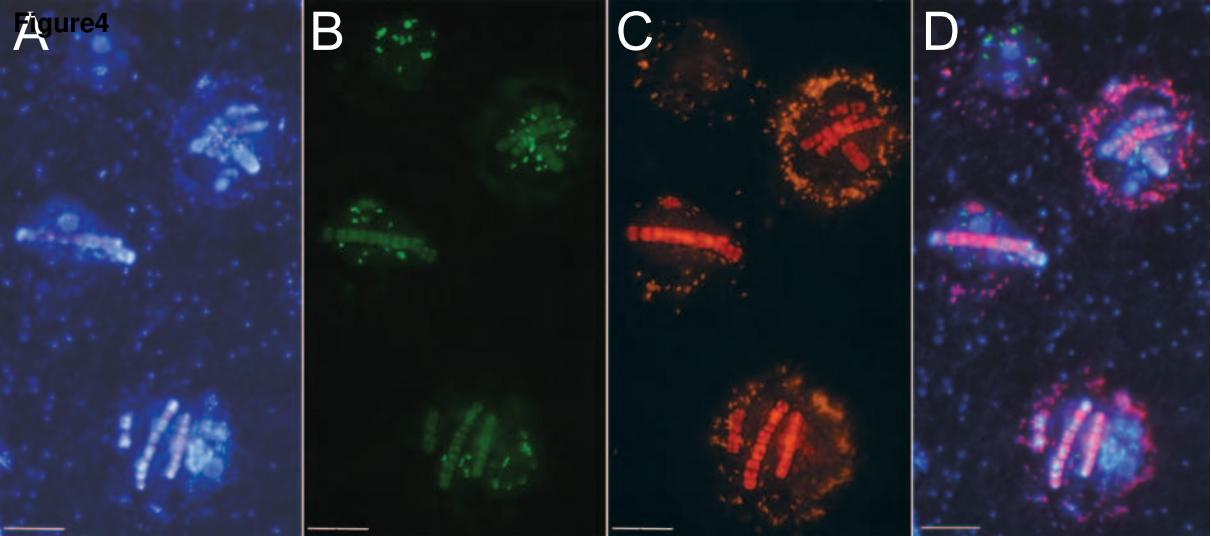


Figure5

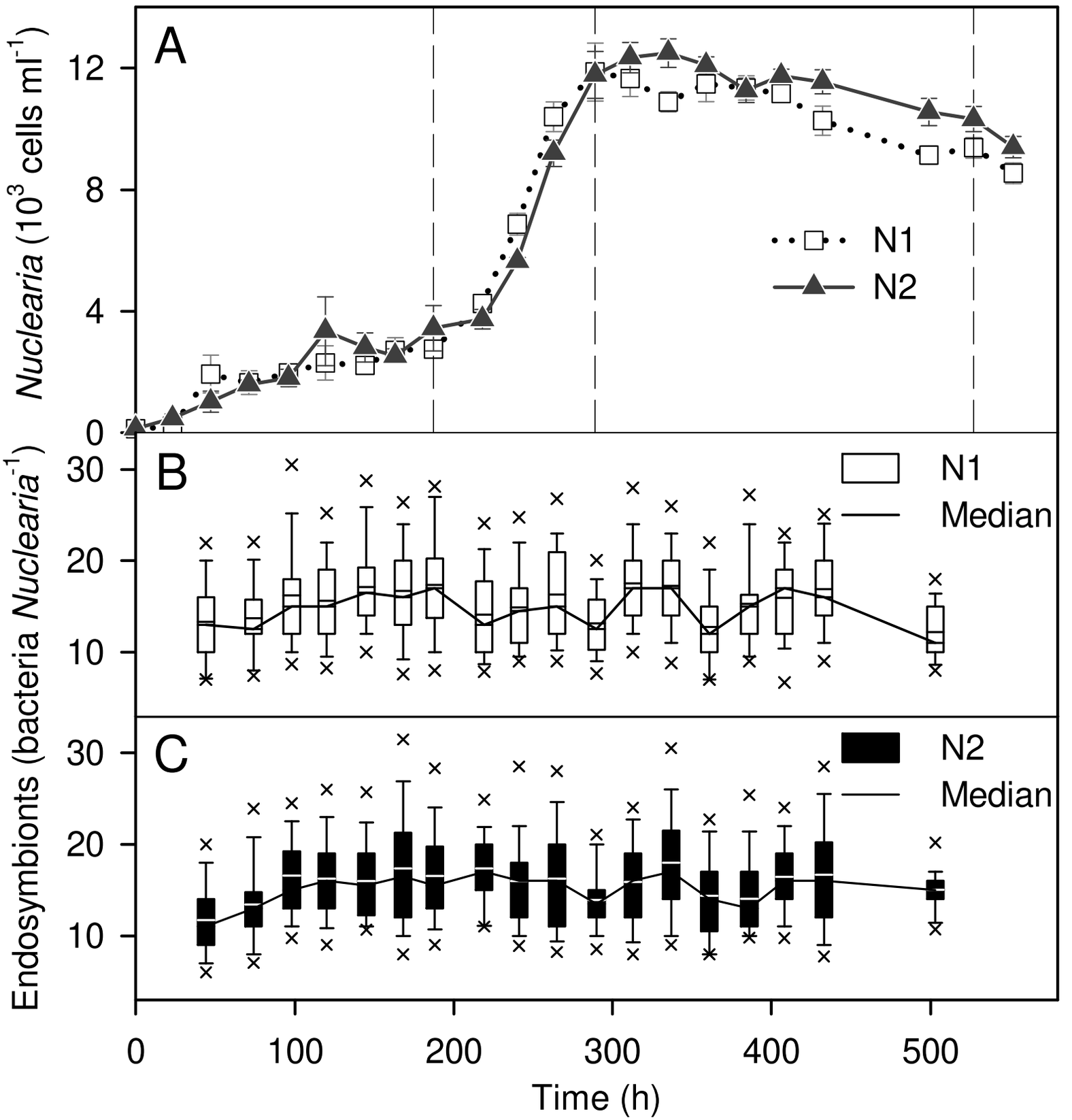
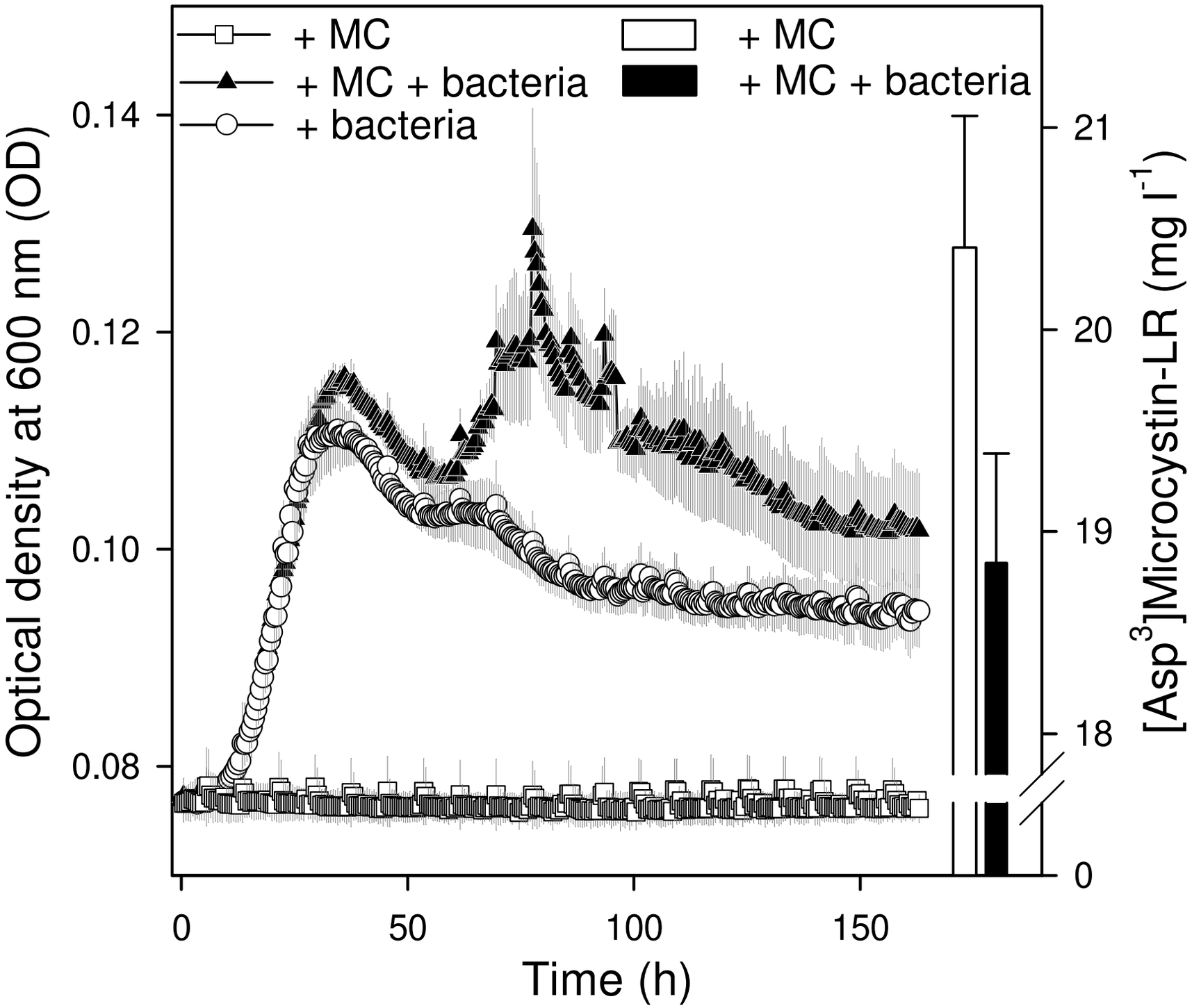

A
B
C
N1
N2
Median
Nuclearia (10^3 cells ml^-1)
Endosymbionts (bacteria Nuclearia^-1)
Time (h)
0
4
8
12
10
20
30
100
200
300
400
500

Figure6


+ MC
+ MC + bacteria
+ bacteria
+ MC
+ MC + bacteria
Optical density at 600 nm (OD)
0.14
0.12
0.10
0.08
21
20
19
18
0
[Asp3]Microcystin-LR (mg l-1)
0
50
100
150
Time (h)